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Receptor Signaling on Mouse Mammary Neoplasia

PRINCIPAL INVESTIGATOR: Michael R. Crowley, Ph.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

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13. ABSTRACT (Maximum 200 Words) <p>Our laboratory has developed transgenic mice that express a zinc-inducible, kinase-defective dominant-negative TGF-β type II receptor in the mammary stroma (DNIIR). Recent evidence has accumulated implicating the stroma in regulating tumor formation. To determine if loss of TGF-β signaling in the stroma affects tumor development transgenic and wild type mice were given pituitary isografts, zinc water and either left untreated or treated with 7,12-dimethylbenz (a) anthracene (DMBA). Fifteen tumors developed in the wild type group on a full regiment (pituitary isograft, zinc and DMBA) while 16 tumors arose in the transgenic group with the same treatment. Both wild type and transgenic mice had an average of 1.5 tumors per mouse with an average latency of approximately 14 weeks. To identify genes regulated by TGF-β in the mammary stroma a filter based array, commercial gene chip microarray and a custom mammary gland specific microarray were screened. Fifty-one genes have been analyzed by RT-PCR and 17 were confirmed for altered expression. The custom mammary gland specific microarray was verified and approximately 40% of the genes were found to be regulated 2 fold above or below the mean. Expression analysis of these genes is currently underway.</p>				
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The transforming growth factor betas (TGF- β 1, - β 2, and - β 3) are members of a family of peptide growth factors that include inhibins, bone morphogenic proteins (BMPs) and growth and differentiation factors (GDFs) (1-3). Activation of the TGF- β receptor results in Smad activation and ultimately to changes in gene expression. TGF- β s have long been associated with diverse cellular processes including but not limited to growth arrest in epithelial cells, cell proliferation and differentiation of mesenchymal cells during development, cell migration in wound healing, extracellular matrix production and immunosuppression. The TGF- β 's and their receptors are expressed throughout the mammary gland where it has a negative effect on the growth of the ductal epithelium and lobuloalveolar development depending on the timing of TGF- β expression. Utilizing antibodies that can discriminate between the active and latent forms of TGF- β 1 Ewan et al., (2002) showed intense active TGF- β 1 staining in the TEBs with more moderate levels in the subtending ducts. The latent form of TGF- β 1 was observed at high levels throughout the epithelium and the fibrous stroma (4). TGF- β signaling in the stroma has been shown to be necessary for proper ductal development in the mammary gland. Expression of a kinase-deficient dominant-negative TGF- β type II receptor (DNIIR) in the mammary epithelium and in stromal fibroblasts resulted in precocious lobuloalveolar development and increased lateral branching, respectively, compared to wild type littermates (5,6). In addition, wild type epithelium transplanted into the cleared DNIIR expressing fat pad resulted in increased ductal branching (our unpublished results). Normal ductal branching was observed in epithelium from DNIIR expressing mice transplanted into wild type fat pads. These data confirm the requirement for the mammary stroma and TGF- β for proper ductal morphogenesis. In mice, several studies support TGF- β signaling as a tumor suppressor for the mammary gland. TGF- β expression inhibited tumor formation when mice expressing an epithelial TGF- β transgene were challenged with 7,12-dimethylbenz (a) anthracene (DMBA) (7). In a separate experiment, DMBA treatment of mice expressing the DNIIR transgene in the mammary epithelium, eliminating TGF- β signaling, increased the incidence and number of tumors (8).

The purpose of this proposal is to test the overall hypothesis that development of mammary tumors will be altered in the absence of TGF- β receptor signaling in the mammary stroma. It is also hypothesized that there are changes in gene expression resulting from the lack of TGF- β signaling in the mammary stroma that will ultimately influence tumor development. To test these hypotheses Serra and colleagues (1999) have engineered two transgenic mouse lines (MTR4 and MTR28) that expressed a kinase-defective dominant-negative TGF- β type II receptor driven by a metallothionein promoter (DNIIR), which allows for regulation of the transgene by heavy metals (e.g. zinc sulfate) (6). Assessing what role, if any, the lack of TGF- β receptor signaling in the mammary stroma affects mammary neoplasia will help to identify novel diagnostic or therapeutic strategies heretofore unexplored.

Previously, Joseph, et al., (1999) had shown that the loss of responsiveness to TGF- β signaling in the mammary stromal fibroblasts resulted in increased lateral branching of the mammary ductal epithelium in adult non-parous animals. Moreover, it has been shown that the mammary stroma may contribute to the generation of experimentally induced mammary tumors (9). Tumor development was compared in wild type and DNIIR (MTR28) transgenic mice (6). As stated in Task 1 of the Statement of Work (S.O.W.), four groups of mice were established. The groups consisted of wild type mice given pituitary isografts and zinc-water, wild type mice with pituitary isografts, zinc-water and carcinogen, MTR28 transgenic mice with pituitary isografts and zinc-water and MTR28 transgenics with pituitary isografts, zinc-water and

carcinogen. When female mice were 5 weeks of age pituitaries from sibling male mice were removed and implanted under the kidney capsule of the recipient female. The mice were placed on zinc-water at 6 weeks of age to induce the transgene or to control for the effects of zinc, if any, on gene expression. The mice were then either treated weekly for 4 weeks with 1mg per week of 7,12-dimethylbenz (a) anthracene (DMBA) beginning at 8 weeks of age or not treated with the carcinogen. Forty-five wild type mice were treated with DMBA in addition to the pituitary isografts and zinc sulfate in the drinking water, 9 mice were lost to lymphoma or leukemia while 15 mammary tumors developed in these mice with an average of 1.5 tumors per mouse and an average latency period of approximately 14 weeks. These mice were on study for an average of 62 weeks with 58.3% of the mice being tumor free at the end of the study period (Fig. 1). This number of mice lost during the study was expected as DMBA induces leukemia and lymphoma in addition to mammary carcinomas. The tumors were analyzed and classified as adenosquamous carcinoma (6 of 15), adenocarcinoma (2 of 15), differentiated squamous carcinoma (2 of 15), less differentiated squamous carcinoma (2 of 15), and adenomyoepithelioma (3 of 15) (Table 1). The majority of tumors that arose in the wild type mice treated with DMBA were adenosquamous carcinoma, which is typical for DMBA initiated tumors in mice. Forty MTR28 mice have received the pituitary isograft, zinc-water and have been fully dosed with carcinogen. Five mice were lost to lymphoma or leukemia during the study period. Sixteen transgenic mice developed tumors, which resulted in 53% of the mice remaining tumor free during the study period (Fig 1.). The mice had an average of 1.4 tumors per mouse with an average latency of 15 weeks. The tumors were categorized as adenosquamous carcinoma (3 of 16), adenocarcinoma (1 of 16), adenocarcinomas with spindle cell formation (2 of 16), differentiated squamous carcinoma (7 of 16), and less differentiated squamous carcinoma (3 of 16). There were no adenomyoepitheliomas detected in the transgenic group treated with DMBA (Table 1). Fifty-nine wild type mice with a pituitary isograft under the kidney capsule and treated continuously with ZnSO₄ in the drinking water for an average of 63 weeks have been analyzed for tumor development. These mice do not carry the DNIIR transgene and therefore have a functional TGF- β response. A total of 9 mice were lost during the study period of undetermined means. Eight tumors arose in the 50 remaining mice resulting in 84% of the mice being tumor free at the end of the study period (Fig 1). The mice had an average of 1.75 tumors per mouse with a latency approaching 60 weeks. Currently, there are 31 MTR28 transgenic mice with pituitary isografts and zinc-water, 18 of these mice were added to the study in March of 2003. These mice have been on the study for approximately 5 months with an additional 13 mice on study for approximately 52 weeks. Three mice in this group have developed tumors with an average of 3 tumors per mouse and a latency of 30 weeks. The histology of the tumors from the pituitary and zinc only groups has not been determined.

In an effort to verify the classification of myoepitheliomas the marker smooth muscle alpha actin (SMA) was used. Tumors derived from DMBA treatment in both wild type and DNIIR expressing mice have been analyzed by immunofluorescence for SMA (Fig. 2). The tumors isolated from the wild type mice and classified as adenomyoepitheliomas by histological analysis stained strongly for SMA throughout the tumor (Fig. 2C). Several tumors of other classifications also stained for SMA expression, however this staining was observed around the glandular or ductal structures within the tumor (data not shown). Tumors isolated from the transgenic mice had very little SMA staining and when present it was found around ductal structures within the tumor (Fig. 2D). Mammary glands from wild type and DNIIR transgenic mice were also analyzed for SMA by immunofluorescence (Fig. 2E and F). The normal ductal

structure has a layer of myoepithelial cells directly adjacent to the epithelial cell layer. Staining for SMA was prominent and continuous around ducts from wild type mice (Fig. 2E). In contrast, SMA staining was reduced and discontinuous around ducts from transgenic mice that expressed DNIIR (Fig 2F). The significance of the lack of SMA seen in the transgenic mice is unknown but loss of myoepithelial cells may precede side branch development. Alternatively, TGF- β signaling in the adjacent fibroblasts may contribute to the maintenance of myofibroblasts.

Analysis of mammary gland tissues sections revealed an increase in eosin staining stroma in the DNIIR expressing mice compared to wild type controls (Fig. 3A and B). An increase in collagen deposition was observed in the DNIIR expressing mammary glands by trichrome staining compared to wild type controls (Fig. 3C and D). Trichrome stains collagen fibers specifically. The increase of stromal collagen was not seen in tumors from DNIIR mice, however (data not shown).

In summary, wild type and DNIIR expressing mice treated with carcinogen did not differ in multiplicity of tumors, the size of the tumors or in tumor latency. Pathological analysis of these tumors did reveal a prevalence of differentiated squamous carcinoma in the DNIIR expressing mice compared to the more typical adenosquamous carcinoma found in DMBA induced tumors. These data suggest that the lack of TGF- β signaling in the mammary stroma may alter the type of tumor derived from chemical carcinogen treatment of mice.

In order to identify genes whose expression was altered by the loss of TGF- β responsiveness in the mammary stroma several different microarrays were screened. As discussed above, in response to zinc the DNIIR mice lack a functional TGF- β response in mammary stromal fibroblasts (6). In contrast, mammary fibroblasts isolated from non-transgenic mice and grown in culture retain a functional TGF- β response. By comparing the expression profiles of genes in the transgenic mammary gland to that from the cells *in vitro*, it is possible to identify genes that are directly regulated by TGF- β in mammary stromal fibroblasts. Using the Incyte gene chip microarrays we had identified 48 known genes and ESTs that were upregulated and 15 genes and ESTs that were downregulated in transgenic mammary glands relative to the mammary glands from wild type mice. Hybridization of the microarrays with RNA isolated from primary mammary fibroblasts treated and untreated with TGF- β resulted in 24 known genes and ESTs identified as up-regulated and 30 genes and ESTs being down-regulated. Recently we have used a combination of semi-quantitative RT-PCR and real time quantitative RT-PCR (QRT-PCR) to verify some of these genes. Semi-quantitative RT-PCR was accomplished by standard RT-PCR of RNA isolated from wild type and transgenic mice on zinc sulfate for 1 and 3 weeks by removing aliquots of the PCR reaction every 5 cycles beginning at cycle 20 (Fig. 4). The genes analyzed in this manner were compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control. Quantitative real time PCR was done using the Cepheid light cycler and sybr green chemistries utilizing 18S rRNA as an internal control. To date 51 genes have been analyzed that represent various biological pathways including lipid metabolism, DNA replication, RNA processing, signal transduction, cell cycle regulation, extracellular matrix and apoptosis. Changes in gene expression between wild type and transgenic mammary glands has been confirmed for 14 of the 51 genes (~30%). Approximately 20 genes have given ambiguous or no results possibly due to faulty primer design. The remaining 17 genes were not regulated in the mammary gland. Figure 4 shows semi-quantitative RT-PCR results for several representative genes. These results demonstrate that lack of TGF- β signaling does indeed have an affect on gene expression in the mammary gland.

Mammary gland specific "gene chips" have been created by suppression-subtraction hybridization (SSH) using RNA isolated from both wild type and DNIIR transgenic mice on zinc for 1 week. Approximately 1,440 clones from both libraries were sequenced by The Cincinnati Children's Hospital Research Foundation genome core facility. To date 576 clones from both libraries have been analyzed by database mining with approximately 40 percent of the sequenced clones being informative. The mammary gland specific gene chips were hybridized in triplicate with cDNA synthesized using RNA isolated from wild type and DNIIR transgenic mammary glands treated with zinc sulfate for 1 week. In total, 1,921 duplicate spots were recognized on the gene chips with approximately 1,000 spots listed as present on all 3 experiments (Fig. 5A). The standard error for each spot was calculated based on replicates within the array and two types of normalization were used to standardize expression levels. After normalization, 399 spots were determined to be regulated with ratios either 2 fold above the mean or 0.5 fold below the mean (Fig. 5B). Currently, individual spots are being analyzed for expression levels in the mammary glands of wild type and transgenic mice. At this time the Incyte gene chip and the Clontech filter array have not been compared to the regulated genes from the custom gene chip to determine overlapping genes. Also, cDNA from primary mammary fibroblasts grown in culture has not been used to screen the custom gene chips.

List of Key Accomplishments

Task 1. Induce Mammary Tumors in MT-DNIIR-28 (MTR-28) and Wild Type Mice, Months 1-24:

- Mice given pituitary isografts, zinc and carcinogen did not have differences in:
 - tumor frequency
 - tumor size
 - latency
- Tumors did vary with respect to histology
 - Adenosquamous carcinomas were the largest group of wild type tumors
 - Differentiated squamous carcinoma was the largest group of tumors from the transgenic group
- The above data suggest that lack of TGF- β signaling in the mammary stroma may affect the type of tumor but not its development.
- Comparisons of tumors from wild type and transgenic mice with pit isografts and zinc only has not been completed because the transgenic group is still on study.

Task 2. Produce and Screen Tissue Specific Microarray “Gene Chips”, Months 18-36:

- Expression analysis of approximately 30% of the genes identified through a filter array and commercial gene chips have been verified.
- Mammary gland specific microarrays have been screened with cDNA from wild type and transgenic mammary glands
- Forty percent of the genes screened on the custom microarrays were regulated.
- Several genes in a diversity of biological pathways are regulated by the loss of TGF- β signaling in the mammary gland.

List of Reportable Outcomes

Presented abstract “Stromal Regulation of Ductal SideBranching in the Mammary Gland: The TGF- β -Cbl Connection” at the 2003 Gordon Conference on Mammary Gland Biology.

Presented abstract “Transforming Growth Factor- β in the Stroma Influences Gene Expression and Development in the Mouse Mammary Gland” at the 2003 Gordon Conference on Mammary Gland Biology.

A review manuscript was submitted to Breast Disease entitled “TGF- β in Mammary Gland Development and Breast Cancer”

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Figure 1. Modified Kaplan-Meyer. Wild type and DNIIR expressing transgenic mice were given a pituitary isograft under the kidney capsule, zinc sulfate in the drinking water, and either treated with DMBA (WT DMBA and MTR28 DMBA) or left untreated (WT Pit and MTR28 Pit) for the study period (52 weeks). The graph shows the percentage of tumor free mice over time. The majority (84%) of wild type mice without carcinogen remained tumor free for the duration of the study. The average latency for tumors in this group was 60 weeks. MTR28 mice without carcinogen are still on the study; however, of the mice that have completed the study 73% are tumor free with tumors arising after 30 weeks. The wild type and transgenic mice with carcinogen treatment did not demonstrate differences in the number of tumor free mice or in tumor latency.

Figure 2. Tumor histology and smooth muscle alpha actin immunofluorescence. A. Histology of a representative adenomyoepithelial tumor derived from the wild type mice treated with DMBA stained with H&E. B. An adenocarcinoma with spindle morphology isolated from a MTR28 transgenic mouse stained with H&E. C. A myoepithelioma stained with an antibody directed against smooth muscle alpha actin (SMA) (red) and counterstained with YoPro (green) to visualize the nuclei. There was a high level of SMA expression in the myoepithelioma. D. A squamous carcinoma isolated from a DNIIR expressing mouse and stained for SMA (red) and counterstained with YoPro (green). Little, if any, SMA staining was visible within the tumors from the transgenic mice. E. Sections of normal mammary gland from wild type mice were analyzed for SMA expression. The mammary ducts (green) are surrounded by a continuous layer of myoepithelial cells (red) that are positive for SMA. F. Mammary ducts (green) from DNIIR expressing mice displayed a discontinuous pattern of SMA expression (red).

Figure 3. DNIIR expressing mice display an increase in fibrous stroma surrounding ductal epithelium. Histological analysis of mammary glands from wild type mice (A and C) compared to transgenic mice (B and D). Sections from mammary glands from wild type (A) and transgenic (B) mice were stained with hemotoxylin and eosin using standard methods. There was an increase in the eosin staining matrix surrounding the mammary duct from the transgenic mouse compared to the wild type mouse. The collagen specific stain, trichrome, was used to determine the extent of collagen deposition in the mammary glands from wild type mice (C) compared to the transgenic mice (D). Trichrome stains the collagen fibers blue. Note the increase in blue trichrome stain surrounding the ducts from the transgenic mouse (D).

Figure 4. Semi-quantitative RT-PCR for a representative subset of genes identified to be regulated in the mammary gland of the transgenic mice and in mammary fibroblasts in culture. Total RNA was isolated from mammary glands of wild type mice (Wild type/Untreated) or transgenic mice (Transgenic/Treated) treated with zinc sulfate in the drinking water for 1 week and from wild type mammary fibroblasts grown in culture and untreated (Wild type/Untreated) or treated (Transgenic/Treated) with 5ng/mL of TGF- β 1 for 8 hours. cDNA was prepared from whole mammary tissue (Mammary gland array and Mammary gland library) or from the cultured cells (Fibroblast array) by standard methods. Aliquots of the PCR reactions were removed every 5 cycles beginning with cycle 15 (i.e. 15, 25, 30, 35 PCR cycles at bottom of figure). Expression of the gene of interest (leptin, platelet derived growth factor receptor alpha [PDGFRa], collagen VI, ErbB3, fibronectin, etc.) was compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control. Note that PDGFRa was increased in the mammary glands of

transgenic mice compared to controls yet it was decreased in the mammary fibroblasts treated with TGF- β 1. These results suggest that PDGFR α is directly regulated by TGF- β .

Figure 5. Scatter plot analysis of the custom mammary gland gene chip microarray. A. Scatter plot analysis of the custom microarray hybridized with total cDNA labeled with Cy3 from the mammary glands of wild type mice and labeled with Cy5 from transgenic mice. The majority of genes were not regulated and fell between the upper line (representing 2 fold above the mean) and the lower line (representing 0.5 fold below the mean) and are false colored in yellow. Genes whose expression were 2 fold above the mean are represented as red and those 0.5 fold below the mean are blue. B. Same as in A except the analysis was restricted to the genes found expressed either 2 fold above the mean or 0.5 fold below the mean. The chips were normalized with Arabdosis genes that were spotted onto the slides and spiked within a sample. A second normalization was done using a non-regulated gene on the array (this was determined by RT-PCR analysis of that particular gene—Fabp4). After normalization 40% of the genes spotted onto the slides were found to be regulated by lack of TGF- β signaling in the mammary glands.

Figure 1.

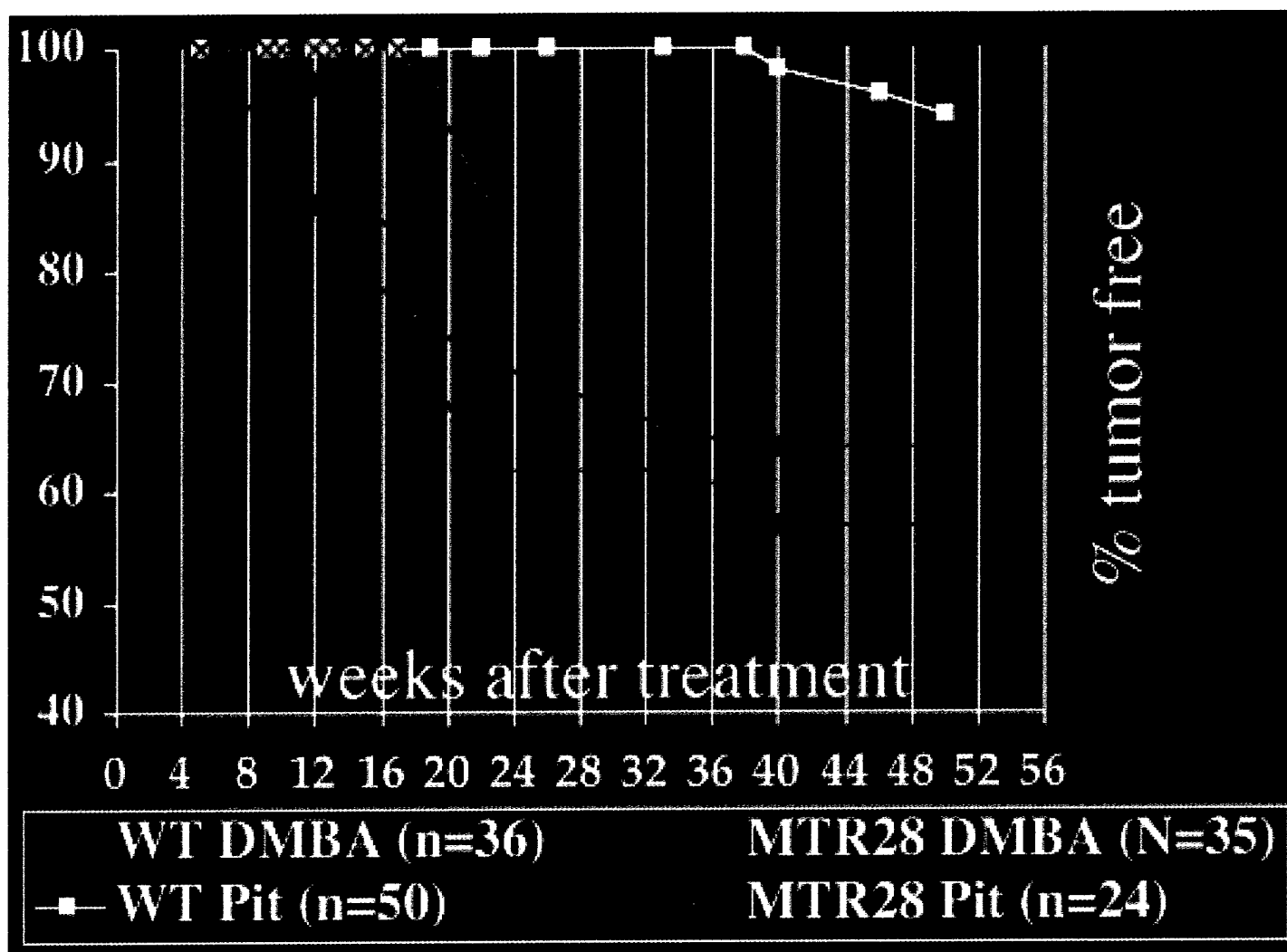


Figure 2.

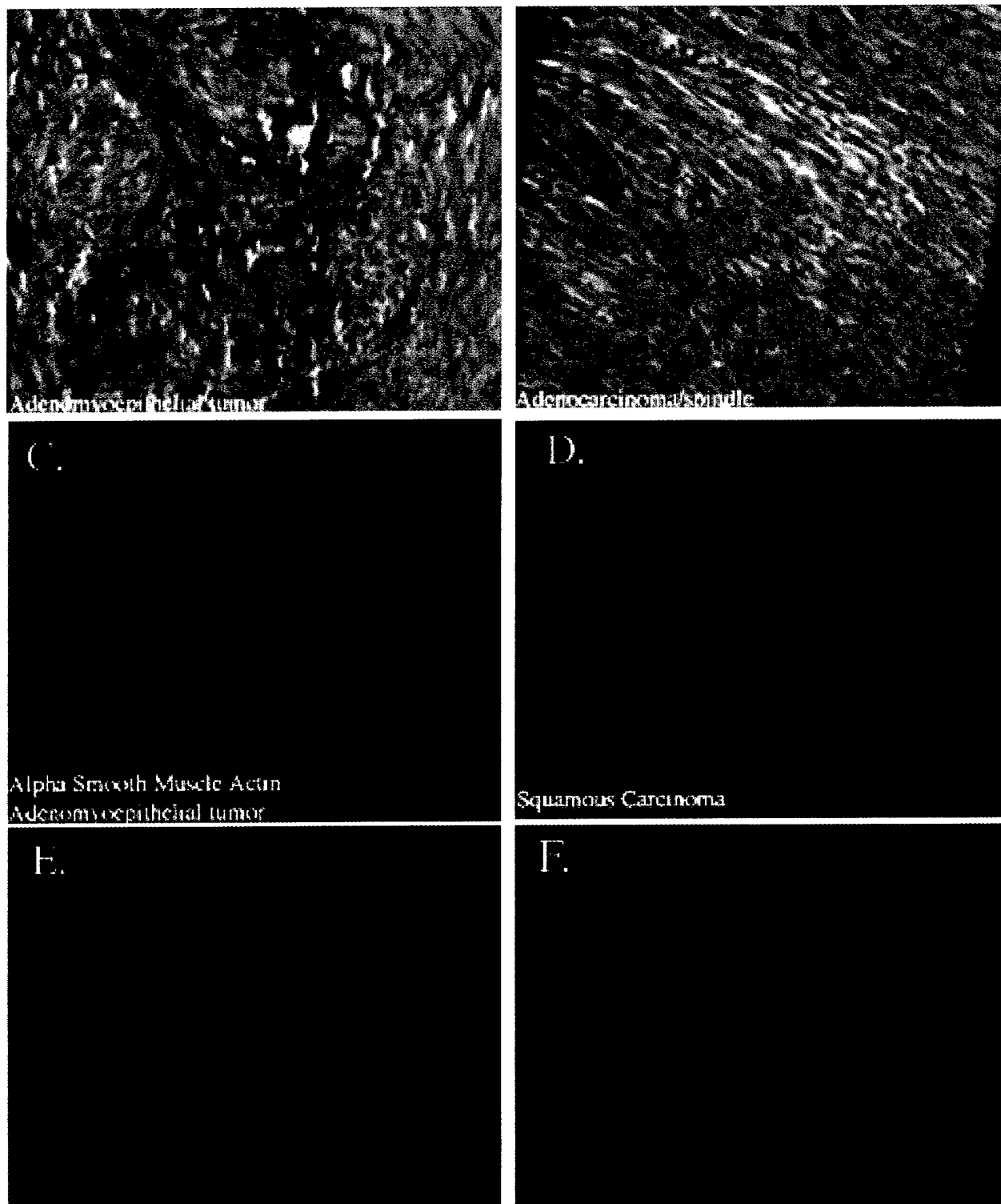


Figure 3.

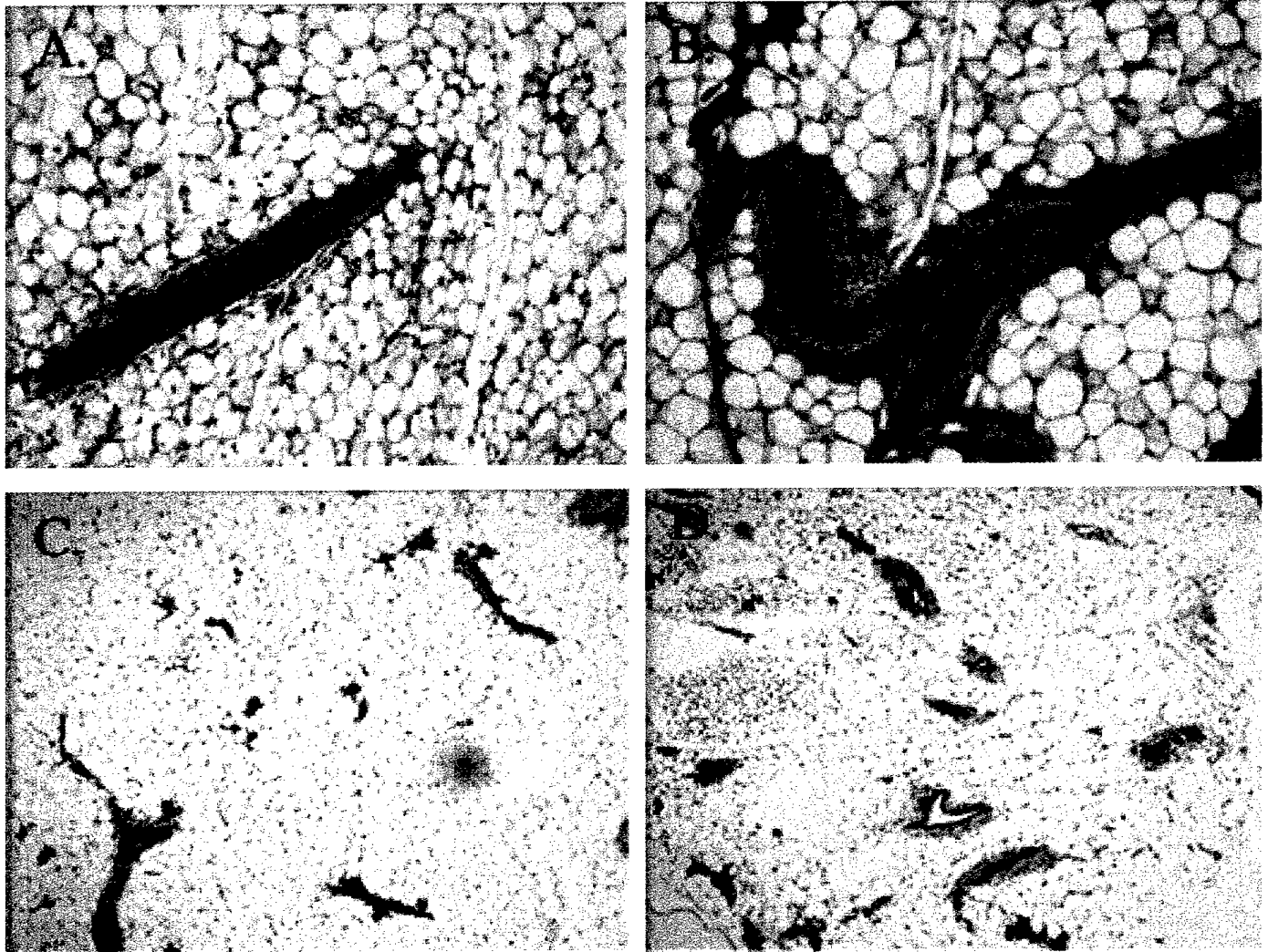


Figure 4.

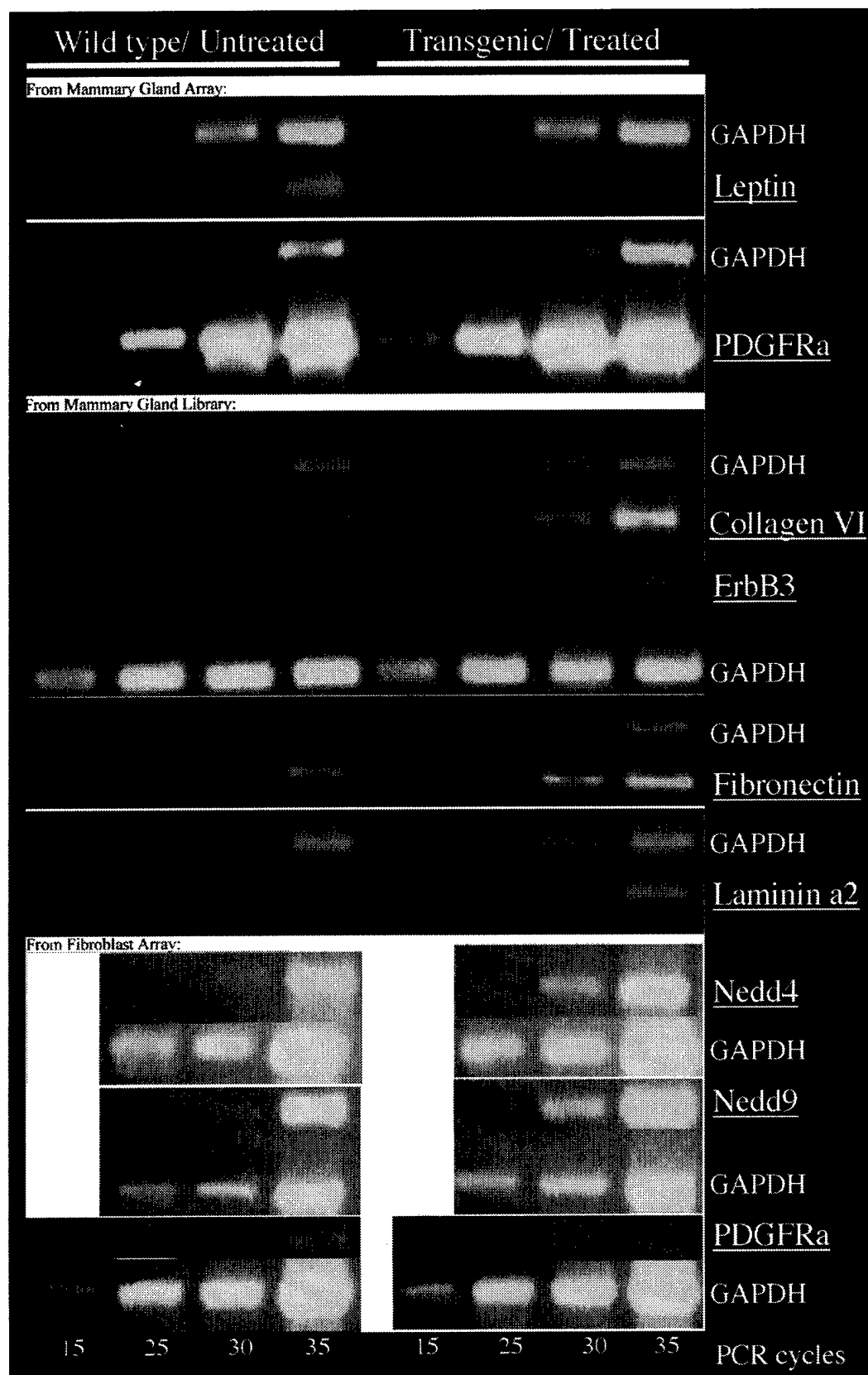


Figure 5.

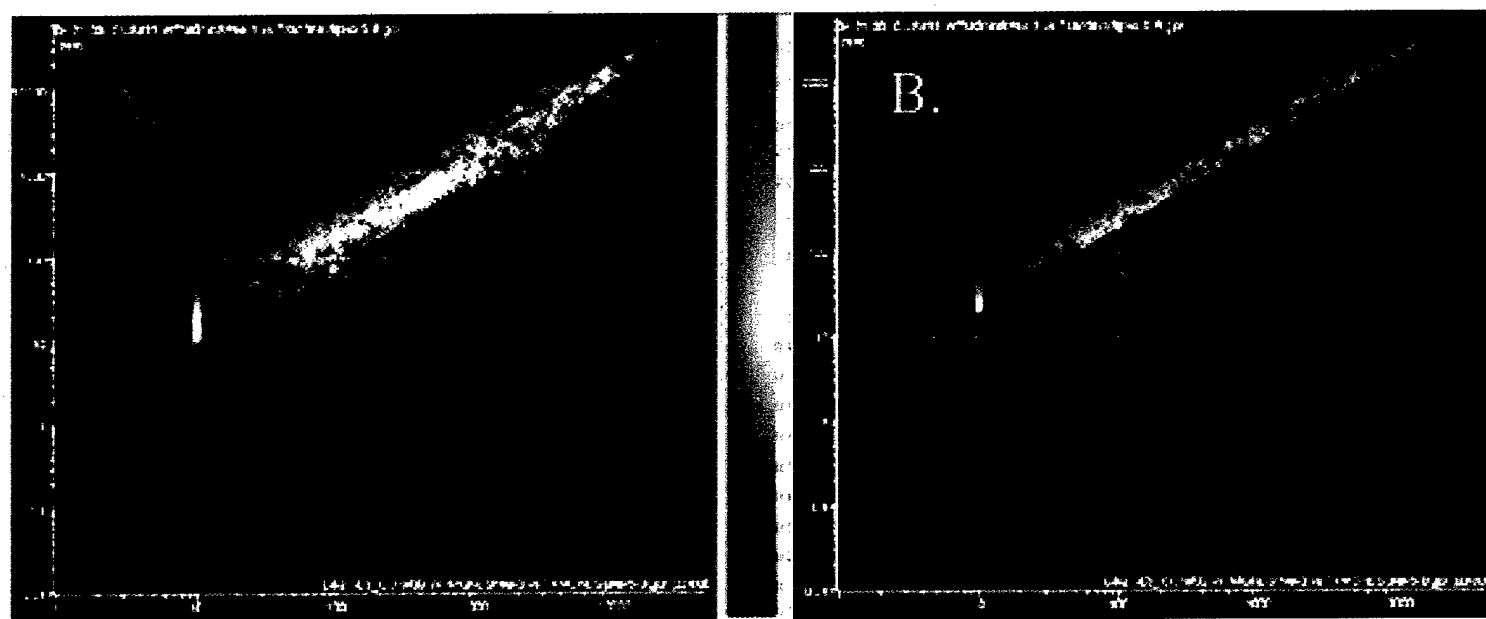


Table 1. Histological analysis of mammary tumors derived from wild type and transgenic mice treated with DMBA.

	WT	MTR28
Adenosquamous	6/15	3/16
Adenocarcinoma	2/15	1/16
spindle	0/15	2/16
Squamous diff	2/15	7/16
less diff	2/15	3/16
Adenomyoeppi	3/15	0/16